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A sulfated chitin inhibits hemagglutination by *Theileria sergenti* merozoites

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Abstract

Five structurally different polysaccharides were examined for their hemagglutination inhibition activity. A (1 → 4)-linked homopolysaccharide composed of *N*-acetyl, 6-*O*-carboxymethyl glucosamine (CM-chitin) showed no inhibitory activity but a 3,6-*O*-sulfated derivative of CM chitin (SCM-chitin III) showed potent hemagglutination inhibition activity. A *N*-sulfated derivative of *N*-deacetylated CM chitin (SCM-chitosan) however, was not effective. Chondroitin sulfate and dermatan sulfate, both of which consisted of disaccharide repeats of (1 → 3)-linked uronic acid and galactosamine sugar residues (→ 4GlcAb1/IdoAa1 → 3GalNAcb1), did not interfere with the red blood cell (RBC) binding by *T. sergenti* merozoite. Thus, the sulfated glycoconjugate interaction is presumed to be an important process in the RBC invasion by the Theilerial merozoite.

Keywords: Theileria sergenti; Erythrocyte invasion; Chitin heparinoid; Hemagglutination

1. Introduction

Theileriosis caused by the infection of a protozoan hemoparasite *Theileria sergenti*, which cannot cultivate in vitro or infect small laboratory animals, is still one of the most economically significant diseases of cattle in Japan, Korea and southern China. The parasite infection by itself develops only mild anemia but the majority of infected animals become long-lasting carriers of this parasite and occasionally develop severe and fatal anemia under unfavorable conditions (Minami, Fujinaga, Furuya & Ishihara, 1980; Kawamoto et al., 1991; Takahashi, 1976). The disease, initiated by invasion, results from repeated proliferations of merozoites in the host's red blood cells (RBC). A few studies on the process of RBC invasion by *T. sergenti*, have been performed at the light microscope level and with the electron microscope (Kawai, Takahashi, Kurosawa & Sonoda, 1993; Kawamoto, Takahashi, Kurosawa, Sonada & Onuma, 1990a; Kawamoto, Takahashi, Onuma, Kurosawa & Sonada, 1990b); however, because of the lack of a suitable assay system, very little is known about the characteristics and the mechanisms involved. We, therefore, developed recently a hemagglutination and a hemagglutination inhibition assay to analyze the adhesion property of *T. sergenti* merozoites to bovine (Bo)-RBCs and, surprisingly, found that the agglutination was inhibited by heparin (Hagiwara, Takahashi, Ichikawa, Tsuji, Ikuta & Ishihara, 1997).

Based on the competitive interference of the host cell–parasite binding by various polysaccharide analogs such as fucoidan, dextran sulfate, heparan sulfate and heparin, several proteineous ligands and carbohydrate residues that are involved in the invasion of RBCs or of hepatocytes by *Plasmodium falciparum* have been identified (Ying et al., 1997; Butcher, Parish & Cowden, 1988; Kulane, Ekre, Perlman, Rombo, Wahlgren & Wahlin, 1992; Sivaraman, 1983). To further characterize the RBC invasion by *T. sergenti*, we examined here the hemagglutination inhibition assay by using three derivatives of chitin, a homopolysaccharide composed of 1 → 4-linked *N*-acetylglucosamine, and two sulfated proteoglycans, chondroitin sulfate (chondroitin sulfate A) and dermatan sulfate (chondroitin sulfate B). The chemical structure of chitin and its derivatives are shown in Fig. 1.

Abbreviations: CM, carboxymethyl; PBS, phosphate-buffered saline; RBC, red blood cell; SCM, sulfated carboxymethyl.

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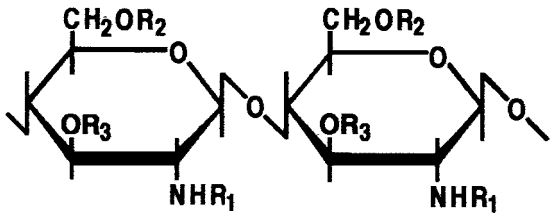

				MW (X10^4)
Chitin	R_1 = $COCH_3$	R_2 = H	R_3 = H	
CM-chitin	= $COCH_3$	= H, CH_2COOH	= H	6.3
SCM-chitin III	= $COCH_3$	= CH_2COOH : 0.56 = SO_3H : 0.44	= SO_3H : 0.60 = H : 0.40	2.4
SCM-chitosan	= $COCH_3$: 0.30 = H, SO_3H : 0.70	= $COCH_3$: 0.56 = H : 0.44	= H	4.7

Fig. 1. Structure of chitin and its derivatives. MW, Average molecular weight was determined from the measured intensic viscosity and the appropriate Mark–Houwick equation.

2. Materials and methods

According to the method described previously (Nishimura, Nishi, Tokura, Nishimura & Azuma, 1986), 6-*O*-(carboxymethyl) chitin (CM-chitin) was prepared from chitin and the degree of substitution was 0.56. The sulfation of CM-chitin was carried out by the general method of Horton and Just described previously (Horton and Just, 1973). Preparation of partially *N*-deacetylated chitin (chitosan), and sulfation of chitosan were carried as previously reported (Nishimura and Tokura, 1987). Hemagglutination and hemagglutination inhibition tests were performed as described previously (Hagiwara et al., 1997). Briefly, blood was withdrawn from a *T. sergenti*-infected calf when its parasitemia developed to about 15%. The RBCs were washed with phosphate buffered saline (PBS, pH 7.2) and were burst using high-pressure nitrogen gas. Free merozoites were obtained by density-gradient centrifugation, followed by washing 3 times with PBS. 50 μl of the purified merozoite suspension which contained 2×10^9 parasites/ml was diluted by two-fold serially in a round-bottomed 96-well microtiter plate. It was then mixed with equal volumes of 2% Bo-RBC suspension and incubated for 1 h at 37°C prior to the determination of agglutination. For the hemagglutination inhibition, test compounds were diluted two-fold serially from 2500 to 19.5 μg/ml and were mixed with equal volume (25 μl for each well) of 5×10^8 merozoites/ml suspension, which was a two fold higher concentration than that required for the complete agglutination. The mixture was then incubated for 1 h at 37°C, added with

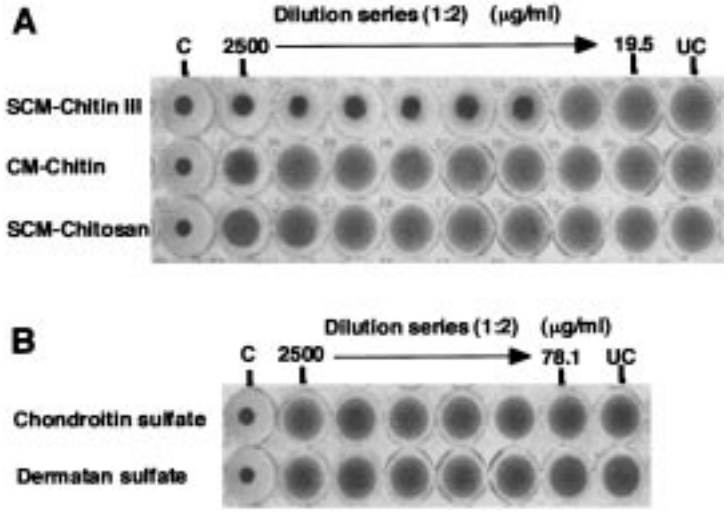

Fig. 2. (a) Hemagglutination inhibition assay with SCM-chitin III, CM-chitin and SCM-chitosan. Two-fold serial dilutions of chitin derivatives were added to the hemagglutination assay. C, no merozoite control. UC, Untreated hemagglutination control (no chitin derivatives). (b) Hemagglutination inhibition assay with Chondroitin sulfate (chondroitin sulfate A) and Dermatan sulfate (chondroitin sulfate B). Two-fold serial dilution of proteoglicans were added to the hemagglutination assay. C, no merozoite control. UC, Untreated hemagglutination control (no proteoglican).

50 μl of 2% bovine RBC suspension and incubated again at 37°C for 1 h prior to the determination for agglutination inhibition.

3. Results and discussion

Here we set out to further characterize the binding property of this parasite by examining five structurally different sulfated polysaccharides—three derivatives of chitin, chondroitin sulfate and dermatan sulfate. The latter two have disaccharide repeats of → 4GlcAb1/IdoAa1 → 3GalNAcb1. A 3,6-*O*-sulfated derivative of CM-chitin (SCM-chitin III) significantly inhibited the hemagglutination by *T. sergenti* merozoites at as less as 78 μg/ml (Fig. 2(a)). This effect seems to be comparable to that of heparin which inhibits the RBC invasion by *T. sergenti* at 200 μg/ml (Hagiwara et al., 1997) and by *P. falciparum* at 200 μg/ml (Xiao, Yang, Patterson, Udhayakumar & Lal, 1996). This interference, however, cannot be based only on the overall sulfate content of the compound, as *N*-sulfated derivative of *N*-deacetylated CM chitin (SCM-chitosan) was as ineffective as the CM-chitin even at 2500 μg (Fig. 2(a)). These in combination with the results showing that neither chondroitin sulfate nor dermatan sulfate exhibited any inhibitory activity (Fig. 2(b)) were indicative that the effect of SCM-chitin III should not be a consequence of nonspecific electrostatic interference but it should be the specific event. In agreement with a number of recent reports that emphasize the crucial role of *O*-sulfation in the interaction of heparin with various growth factors (Arai, Parker, Busby & Clemmons, 1994; Aviezer et al., 1994), it is likely that the (1 → 4)-linked oligosaccharides composed of *N*-acetyl,3,6-*O*-sulfated glucosamine in the structure preferentially interfere with the merozoite-RBC interaction. Sulfated glycoconjugates are implicated as receptors in the hepatocyte invasion by malaria sporozoite. Heparin, dextran sulfate and heparan sulfate are all shown to interfere with the binding of the circumsporozoite protein to heparan sulfate proteoglycans of hepatocyte with different efficiencies (Frevert, Sinnins, Cerami, Shreffler, Takacs & Nussenzweig, 1993), though their structural heterogeneity made it difficult to deduce the underlying characteristics required for the target cell binding. A homopolysacharide, fucoidan, has selectively been examined for the role of *O*-sulfation (Frevert et al., 1993; Pancake, Holt, Mellouk & Hoffman, 1992), and to date the two conserved regions, I and II-plus, of circumsporozoite protein are demonstrated to interact with the unique highly *O*-sulfated, heparin-like oligosaccharides in liver heparan sulfate chains (Ying et al., 1997). RBC invasion by malaria merozoite is shown to be mediated by the specific binding of the lectin-like proteins on the merozoite surfaces to the carbohydrate receptors of RBCs (Jacobson and Doyle, 1996). Three merozoite surface proteins of *P. falciparum* have been identified that are specific for *N*-acetylglucosamine binding and have been designated Pf120, Pf83 and Pf45 (el-Moudni, Philippe, Monsigny & Schrevel, 1993). Moreover, promiscuous lectin-like interactions and their interference by sulfated polysaccharides have also been described in *P. falciparum*-infected RBCs (Carlson and Wahlgren, 1992; Rogerson, Chaiyaroj, Ng, Reeder & Brown, 1995). The present study extended the previous observation and demonstrated for the first time that the RBC binding of *T. sergenti* merozoite could significantly be interfered by sulfated homopolysaccharide, SCM-chitin III, which is composed of 3, 6-*O*-sulfated 1 → 4-linked *N*-acetylglucosamine, though the exact structure and composition of the smallest binding oligosaccharide from SCM-chitin III still require characterization.

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